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RESEARCH PAPER

Polymorphism in miR-31 and miR-584 binding site in the angiotensinogen gene differentially influences body fat distribution in both sexes

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Abstract Angiotensinogen (AGT), its active fragments and microRNA-31 (miR-31) play an important role in adipocyte differentiation. AGT contains a miR-31 polymorphic binding site. We hypothesize that the rs7079 polymorphism in the miR-31/584 binding site of the AGT gene could influence body fat distribution. A total of 751 subjects (195 men, 556 women) were enrolled in the study. The rs7079 genotypes were determined by qRT-PCR. Anthropometric measurements were taken on all subjects, who were subsequently divided into two groups: obese (>30 kg m^{-2}) and non-obese (<30 kg m^{-2}). Linear regression models were created to determine the contributions of sex, obesity status and rs7079 to all measured parameters. Adding the rs7079 genotype significantly contributed to the linear regression model for waist circumference ($p = 0.013$), hip circumference ($p = 0.018$) and supraspinal skin-fold thickness ($p = 1 \times 10^{-3}$). Differences between sexes and between the obese and non-obese groups were observed. Waist circumference was lower in men carrying the A allele ($p = 0.022$); hip circumference was higher only in obese women carrying the A allele ($p = 0.015$). While men carrying the A allele had lower supraspinal skin-fold thickness ($p = 0.022$), this parameter was found to be higher in A allele carrying women ($p = 3 \times 10^{-3}$). The higher total sum of skin-fold thickness in A allele carrying women was restricted to obese individuals ($p = 0.028$). The presence of the A allele was associated with both lower tricipital skin-fold thickness in non-obese women ($p = 0.023$) and a trend of higher thickness in non-obese men ($p = 0.065$). Significant associations of rs7079 in the AGT gene and body fat distribution were observed. The distribution followed opposing patterns in both sexes.

Keywords microRNA · 3′-UTR polymorphism · Genetic variability · Rs7079 · Adipose tissue

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Introduction

The genetic risk factors for obesity have been extensively investigated in many studies. Its heritability is mostly estimated to be slightly over 50 %, with estimates ranging from 20 to 90 % depending on physical activity (Silventoinen et al. 2009), studied population age and study design (Elks et al. 2012; Waalen 2014). Thus far, genome-wide association studies (GWAS) have identified only a small fraction of total heritability (Waalen 2014; Wang et al. 2014) and the predictive value of a genetic scoring system consisting of both GWAS-identified and candidate gene-based approach-identified variants previously associated with obesity has been found to be inferior to conventional risk factors; moreover, adding genetic factors did not improve the predictive value of conventional factors-based model (Morandi et al. 2012). While transgenerational epigenetic effects are likely to play a significant role (Waalen 2014; Kelly et al. 2014), the lack of success in the search for the genetic background of obesity also calls for the

identification of new candidate genetic variants in pathways regulating appetite, energy expenditure and adipose tissue metabolism even in the GWAS era. With increasing knowledge regarding the involvement of micro-ribonucleic acid (miRNA, miR) in adipose tissue metabolism and the potential implications of this knowledge for obesity, polymorphisms of miRNA binding sites of target genes are wort examining. In total, 87 GWAS-identified single-nucleotide polymorphisms (SNPs) associated with disease traits were found to be in a very strong linkage disequilibrium (LD) with functional polymorphisms creating or abrogating miRNA binding sites (Richardson et al. 2011).

MicroRNAs are small non-coding ribonucleic acids post-transcriptionally regulating gene expression. Unlike in many other species, including primates, known human miRNA coding sequences are located mostly in the introns of other genes; however, miRNAs coded in intergenic regions have been more frequently associated with human diseases (Ghorai and Ghosh 2014). Approximately 40 miRNAs have been found to influence adipogenesis (Son et al. 2014) and many miRNAs contribute to the regulation of inflammation in white adipose tissue (Ge et al. 2014). This group includes miR-31, which is produced by adipocytes and its expression has been found to increase after treatment by a macrophage LPS-conditioned medium, mimicking the inflammatory state (Ortega et al. 2015). miR-31 also regulates adipocyte differentiation (Sun et al. 2009), and its regulation of the angiotensinogen (AGT) gene expression, depending on its nucleotide sequence, may represent one of the possible mechanisms (Mopidevi et al. 2013).

Angiotensinogen and its metabolites belong to a group of signalling molecules regulating adipocyte differentiation and proliferation. AGT is extensively produced in adipose tissue, which is responsible for 30 % of its plasma concentration in laboratory rodents (Massiéra et al. 2001). In rodent models, angiotensin II contributes to adipose tissue proliferation by binding on both AT1 and AT2 receptors (Kouyama et al. 2005; Yvan-Charvet et al. 2009), which is opposed by angiotensin 1–7 acting on Mas receptors (Santos et al. 2008). Although data about AGT expression in subcutaneous adipocytes of obese and non-obese human patients remain controversial (Dusserre et al. 2000; Yasue et al. 2010; Okada et al. 2010; Oberbach et al. 2014; Van Harmelen et al. 2000a, b), the plasma levels of circulating angiotensinogen have been found increased in obese patients (Yasue et al. 2010) and they were shown to decrease with the weight loss (Engeli et al. 2005; Oberbach et al. 2014).

Genetic variants of the AGT gene have been repeatedly associated with a visceral pattern of obesity and metabolic syndrome in different populations (Takakura et al. 2006; Procopciuc et al. 2010). The AGT secretion has been found to be influenced by regulatory polymorphisms of the *AGT* gene, as is +11525 C/A (rs7079) in miR-31 and another miRNA, miR-584, binding site. This polymorphism is situated in the seed binding sequence in the 3′-UTR of the AGT gene, which is crucial for binding the two miRNAs which decrease gene expression by post-transcriptional mechanisms. While the C allele is fully complementary with miR-31 and miR-584, the binding is impaired in the case of the A allele. In a study by Mopidevi et al., this resulted in the lower expression of AGT gene containing +11525 C/A (rs7079) C allele than A allele after miR-31 and miR-584 transfection in human kidney and human liver cells (Mopidevi et al. 2013). This SNP has also been linked to hypertension in a Saudi Arabian population (Al-Najai et al. 2013). We thus hypothesize that this polymorphism could also influence fatty tissue distribution patterns.

Materials and methods

Patient selection and anthropometry

The total number of 751 participants (556 women and 195 men) was involved in the study. Participants were enrolled in a mass media campaign addressing people from the South Moravian Region of the Czech Republic. The study was approved by Ethical committee of Masaryk University and was held in accordance with the Declaration of Helsinki.

After a routine medical history acquisition and internal examination (Rihacek et al. 2013), the following anthropometric parameters were measured: waist circumference, waist–hip ratio (WHR), sum of skin-fold thickness in different body regions (triceps, biceps, subscapular, suprasspinal), weight (kg) and height (cm). All of these parameters were measured by an educated specialist using a precisely calibrated set of scales. Body mass index (BMI) was calculated from weight and height using the following formula: $\text{BMI} = \text{weight [kg]}/(\text{height})^2\ [\text{cm}^2]$. Body fat percentage (% fat) was measured by means of a bioimpedance method using InBody device (InBody CO, Ltd., Seoul, South Korea). All measurements were taken in light outdoor clothes in the morning after drinking one glass of water. According to BMI, participants were classified as either obese ($\text{BMI} > 30\ \text{kg m}^{-2}$; $n = 434$) or non-obese ($\text{BMI} < 30\ \text{kg m}^{-2}$; $n = 317$).

Genotyping

All study subjects were genotyped for the +11525 C/A polymorphism of the AGT gene (rs7079). A total of 5 ml of peripheral blood was collected into EDTA tubes. Blood

was gently mixed and frozen at −20 °C prior to DNA isolation. DNA was isolated using the standard proteinase-K technique. The genotyping of selected SNPs was performed using 5′ exonuclease (TaqMan®) reagents using the StepOne® real-time PCR system (Thermo Fisher Scientific Inc., Waltham, USA). The validity of results was confirmed by a second genotyping of 10 % of randomly selected samples; genotypes were confirmed in all cases (100 % match between primary and secondary sampling).

Statistical analysis

By comparing linear regression models, it was determined whether adding the *AGT* +11525 C/A genotype improved the prognostic value of sex and obesity status for parameters assessing body fat distribution; i.e. the model containing sex, obesity status and *AGT* +11525 C/A genotype (grouped as CC homozygotes and A allele carriers) as factors was compared with a model containing only obesity status and sex. An *F* test comparing the distribution of residuals in both models was used for the analysis.

In the explorative part of the analysis, the precise interaction between sex, obesity status and *AGT* +11525 C/A genotype was determined. Prior to that, stepwise linear regression model was computed to see which of the above-mentioned factors were significant determinants of the given parameter of body fat distribution; if a factor was insignificant, it was not included in subsequent exploratory analysis. The exploratory analysis itself was carried out using *t* tests comparing different groups defined by identified parameters.

Results

The study subjects represented various age groups with a mean age of 48 ± 14 years and a predominant share of women (nearly 75 %). Average BMI (31.8 kg m^{-2}) was in the obesity range and nearly half of the study population ($n = 321$) consisted of obese women. All basic study subject characteristics are included in Table 1.

Adding the *AGT* +11525 C/A genotype significantly improved the predictive value of the linear regression model in the case of waist circumference ($p = 0.013$), hip circumference ($p = 0.018$) and supraspinal skin-fold thickness ($p = 1 \times 10^{-3}$). In the case of tricipital skin-fold thickness and the sum of skin-fold thicknesses, the difference between the two models was of marginal significance ($p = 0.056$ for the former, $p = 0.050$ for the latter parameter). The effect of *AGT* +11525 C/A was insignificant in other parameters of body fat distribution.

In a detailed analysis (Table 2), the effect of *AGT* +11525 C/A was found to differ between men and women involved in the study. While waist circumference was significantly lower in men carrying the A allele ($p = 0.022$), no significant association was found in the case of women. Hip circumference was, on the other hand, higher only in obese women carrying the *AGT* +11525 A allele ($p = 0.015$).

Table 1 Basic characteristics of the subjects

Parameter (unit)	Value
Number of study subjects (men)	751 (195)
Age (years)	47.84 ± 14.13
Height (cm)	167.84 ± 8.89
Weight (kg)	89.73 ± 22.13
BMI (kg m^{-2})	31.83 ± 7.38
Obesity (*n*/%)	434/58 %
Systolic blood pressure (mm Hg)	131 ± 20
Diastolic blood pressure (mm Hg)	85 ± 12
Waist circumference (cm)	102 ± 19
Hip circumference (cm)	113 ± 14
Waist/hip ratio	0.89 ± 0.10
Skin-fold thickness—triceps (mm)	25.1 ± 8.0
Skin-fold thickness—biceps (mm)	18.4 ± 8.0
Skin-fold thickness—subscapular (mm)	25.7 ± 9.7
Skin-fold thickness—supraspinal (mm)	22.8 ± 9.1
Skin-fold thickness—sum (mm)	91.5 ± 30.0
Body fat (%)	37 ± 10.4
AGT +11525 C/A genotypes (CC/CA/AA)	375/303/73

The association of skin-fold thickness with obesity status and the *AGT* +11525 C/A genotype showed opposing patterns in men and women in tricipital and supraspinal skin-fold thickness as well as the sum of skin-fold thicknesses. While men carrying the A allele had lower supraspinal skin-fold thickness ($p = 0.022$) compared to CC homozygotes, it was higher in *AGT* +11525 A carrying women ($p = 3 \times 10^{-3}$). The same trends were present in the case of total sum of skin-fold thickness, where, however, the higher thickness in A carrying women was only restricted to obese individuals ($p = 0.028$); in men, the obese A allele carriers showed an insignificant trend towards lower thickness in A carriers compared to CC homozygotes. For tricipital skin fold, the presence of the A allele was associated with a lower thickness in non-obese women ($p = 0.023$) with a higher thickness trend present in non-obese men ($p = 0.065$).

Discussion

The main findings of the study constitute evidence that +11525 C/A (rs7079) in miR-31 and miR-584 binding site of the *AGT* gene influences body fat distribution, while the

Table 2 Effects of AGT +11525 C/A genotype, sex and obesity on body fat distribution

Parameter of body fat distribution	Obesity a significant factor	Obesity status (if applicable)	Men—effect	Men—*p* value	Women—effect	Women—*p* value
Waist circumference	No	NA	**CC > CA + AA**	**0.022**	CC < CA + AA	0.109
Hip circumference	Yes	Obese	CC > CA + AA	0.120	**CC < CA + AA**	**0.015**
		Non-obese	–	–	–	–
Skin-fold thickness (triceps)	Yes	Obese	–	–	–	–
		Non-obese	CC < CA + AA	0.064	**CC > CA + AA**	**0.023**
Skin-fold thickness (supraspinal)	No	NA	**CC > CA + AA**	**0.022**	**CC < CA + AA**	**0.003**
Skin-fold thickness (sum)	Yes	Obese	CC > CA + AA	0.081	**CC < CA + AA**	**0.028**
		Non-obese	–	–	–	–

NA not applicable. Trends with $p < 0.15$ are listed in the table. Significant effects with $p < 0.05$ are marked bold. In case of waist–hip ratio, bicipital and subscapular skin-fold thickness, AGT +11525 C/A genotype was not significant factor in the model

effects show opposite trends in both sexes. Although the evidence of interactions of *AGT* genetic variants with the obesity pattern thus far remains limited, a Hong Kong linkage study comprising 126 families revealed the significant impact of selected *AGT* polymorphisms on WHR without affecting patient BMI (Fang et al. 2010). A study made by our group revealed the effect of *AGT* genetic variants (rs4762, rs699 and rs5051) on BMI in hypertensive patients; however, the distribution patterns of body fat were not investigated (Vasků et al. 2002). The only study so far evaluating the effect of miRNA binding site +11525 C/A (rs7079) on BMI was performed by Al-Najai et al. In accordance with our results, no association with BMI was found; however, the polymorphism was associated with hypertension and coronary artery disease. +11525 C/A (rs7079) was also found to be in LD with other polymorphisms in the *AGT* gene (Al-Najai et al. 2013). Other studies focused on other polymorphisms of the AGT gene that are in LD with rs7079 (International HapMap Consortium 2003), with similar results. The most frequently investigated polymorphism, i.e. M235 exonic SNP (rs699), has been associated with body fat percentage and waist circumference but not BMI or WHR in Japanese women. Similar results were observed in Romanian female population, where rs699 was significantly associated with waist circumference, while the effects on BMI were only marginal (Procopciuc et al. 2010). This was not confirmed for men in a larger Italian study ($n = 959$); however, the I/D polymorphism in the ACE gene was associated with waist circumference and its change during a 20-year follow-up, suggesting the importance of the renin–angiotensin system in this respect. Rs699 and −6 A/G (rs5051) promoter polymorphisms in the AGT gene were also associated with the weight gain of Japanese children treated by glucocorticoids, with both SNPs being in strong LD (Nakamura 2015).

Many miRNAs, including miR-31, have been found to be differentially expressed during the process of adipogenesis (Son et al. 2014; Tang et al. 2009). miR-31 inhibited the differentiation of murine adipocytes from mesenchymal stem cells (MSCs) (Sun et al. 2009), and its expression by MSCs has been found to be downregulated within adipogenesis in a Chinese study (Tang et al. 2009). Moreover, its production is increased in mature adipocytes during inflammation, suggesting the negative feedback during obesity-induced inflammation (Ortega et al. 2015). Among potential molecular targets, phosphoinositide 3-kinase, class 2, alpha polypeptide (PIK3C2A) and CCAAT/enhancer-binding protein α (C/EBPα) have been suggested as mediators of miR-31's action on adipocyte precursors (Tang et al. 2009); the latter of the two has been found to be downregulated by miR-31 in murine mesenchymal stem cell line C3H10T1/2 that differentiates into adipocytes (Sun et al. 2009). To date, no effect of mi-584 on adipocyte differentiation has been reported.

More recently, miR-31 and miR-584 were found to influence the expression of angiotensinogen, with an important effect of +11525 C/A (rs7079) genotype: the downregulation of *AGT* gene expression by these miRNAs was evident in the presence of the C allele but not the A allele, AGT being more expressed in the latter (Mopidevi et al. 2013). Our study indirectly supports the notion that the action of miR-31 (and miR-584, eventually) on AGT gene expression may influence adipocyte growth and differentiation in humans and thus affect adipose tissue distribution (Fig. 1). In subjects with the C allele, AGT expression is decreased, which may eventually result in lower AGT levels. Similarly to AGT, miR-31 was shown to be expressed both in adipose tissue (Tang et al. 2009) and in the liver, where the expression of miR-31 is higher in men than in women (Rieger et al. 2013). The "physical contact" between miR-31 and angiotensinogen may thus occur both

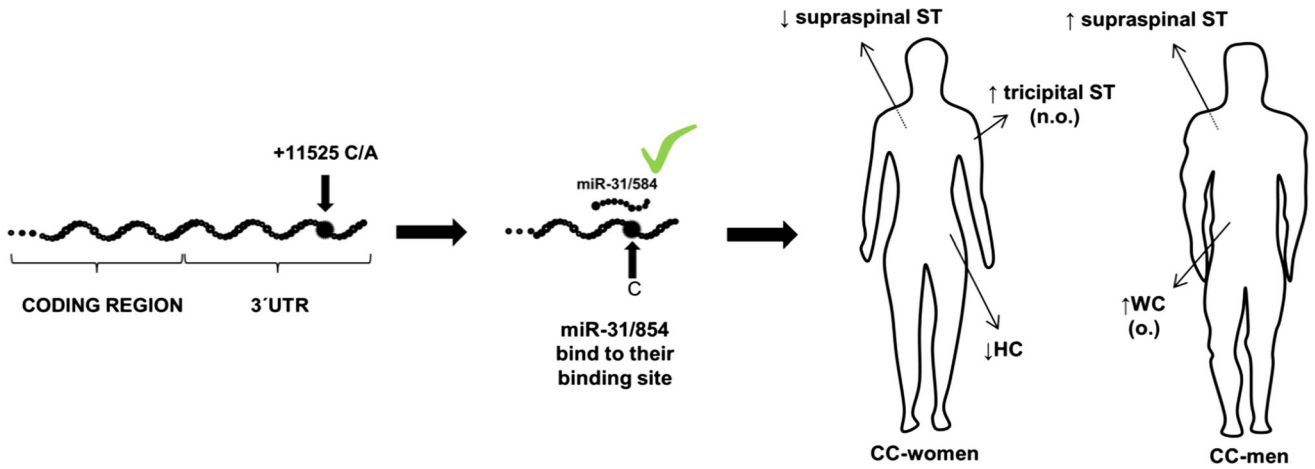

Fig. 1 +11525 C/A (rs7079) polymorphism and body fat distribution. Summary of the effect of the +11525 C/A (rs7079) polymorphism in the angiotensinogen gene on miRNA-31 and miRNA-584 binding effect of the studied polymorphism on body fat distribution in men and women. Only significant results ($p < 0.05$) are shown for the CC allele carriers, and *arrows* (↓/↑) are used to compare CC to CA + AA carriers. *UTR* untranslated region, *miR* microRNA; *ST* skin-fold thickness; *WC* waist circumference; *HC* hip circumference; (*o.*) effect observed only in obese individuals; and (*n.o.*) effect observed only in non-obese individuals

within hepatocytes and differentiating adipocytes. As miR-31 alters the AGT signalling ratio, this may result in altered adipogenesis, which is consistent with the findings of our study. However, studies of gene expression in adipocyte cell culture are needed to confirm this hypothesis; eventually, the observed association could also arise from LD with other, causative polymorphisms (Al-Najai et al. 2013). Using HapMap (International HapMap Consortium 2003) and GTEx, database of expression quantitative trait loci (eQTL) (GTEx Consortium 2013), three polymorphisms in the AGT gene in significant LD with rs7079 were found to be associated with the level of AGT expression in subcutaneous adipose tissue—rs5050 ($D' = 1.0$; 95 % CI = 0.58–1.0; $r^2 = 0.06$), rs11122577 ($D' = 1.0$; 95 % CI = 0.66–1.0; $r^2 = 0.08$) and rs2478545 ($D' = 1.0$; 95 % CI = 0.74–1.0; $r^2 = 0,12$). The promoter polymorphism −20 C/A (rs5050) is a functional SNP; it affects binding of upstream stimulating factor 2 (USF2) leading to the higher transcription of minor C allele containing gene compared to its A allele containing counterpart (Park et al. 2013). Likewise, in the other two polymorphisms, the minor allele homozygotes and heterozygotes have significantly higher expression compared to the major allele homozygotes. As AGT-increasing +11525 C/A (rs7079) A allele (Mopidevi et al. 2013) is only present in combination with the major allele of the three eQTL, the effects on AGT secretion are apparently contradictory and rs7079 CC homozygotic genotype responding to miR-31 and miR-584 binding thus compensate the overproduction of AGT caused by promoter variant. This LD can also explain the findings of Al-Najai et al. where the C allele (G in antiparallel strain) and CC genotype were associated with a higher risk of hypertension (Al-Najai et al. 2013).

In our study, non-obese men with the minor A allele tended to accumulate body fat in the tricipital skin fold, while the amount of their body fat decreased in lower body parts. Opposite effects were observed in women. Provided that the effect of the +11525 C/A (rs7079) polymorphism is causative, this would point to the possibility that angiotensinogen is differentially expressed in both sexes and in different parts of the body. Men generally tend to accumulate body fat in upper and women in lower parts of the body (Palmer and Clegg 2015); both sexes also differ in the metabolism of adipose tissue (Szymańska et al. 2012). This pattern may be partially attributed to local differences in angiotensinogen production by adipocytes (Dusserre et al. 2000; Van Harmelen et al. 2000a, b), which is suppressed by miR-31 and/or miR-584 in C allele homozygotes but not in A allele carriers. Such sex-specific differences in angiotensinogen expression have been confirmed in the case of *AGT* promoter polymorphism −20 C/A (rs5050) when transgenic mice producing human AGT was used (Park et al. 2012). Interestingly, these differences were also depot specific with differences between omental and perigenital fatty tissue. The sex differences were also replicated in human fatty tissue (Park et al. 2013) and may be linked to sex hormone regulation of AGT expression, which has been confirmed in laboratory rodents (Stavréus-Evers et al. 2001; Ding et al. 2001). The results of our study suggest that similar effects can be present in humans in the case of the +11525 C/A (rs7079) polymorphism in miR-31 and miR-584 binding site of the angiotensinogen gene; sex-related differences in miRNA expression observed in the case of miR-31 (Rieger et al. 2013) can be a contributing factor.

Our study has several limitations. First, the study group was relatively small. As participants were enrolled by means of a media campaign, the study group thus consists of highly selected subjects that were interested in campaign; most notably, men make up only 26 % of the group (195 individuals), which decreases the power of the statistical tests performed in the men subgroup. Furthermore, we did not have data on AGT serum level and the interpretation of results is thus based on currently available literature and databases focusing on gene expression rather than on direct data. Finally, due to the monocentric character of the study, the results need replication using different populations.

Conclusion

This study established a significant contribution of the +11525 C/A (rs7079) polymorphism in miR-31 and miR-584 binding site of the *AGT* gene to body weight distribution, in addition to the effects of sex and obesity status. The distribution followed opposite patterns in both sexes. While in men, CC homozygotes tended to accumulate body fat in waist and lower parts of the body compared to A allele carriers, and in women, the effect exhibited an opposite trend, with CC homozygotes having higher tricipital skin-fold thickness compared to A allele carriers. Some of these effects were apparent only in obese or only in non-obese subjects.

Conflict of interest All authors declare that they have no conflict of interest.

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